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Chloroplast endoribonuclease p54 involved in RNA 3′-end processing is regulated by phosphorylation and redox state

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ABSTRACT

Chloroplast RNA-binding protein p54 is an endoribonuclease required for 3′ end-processing of plastid precursor transcripts. We find that purified p54 can serve as a phosphate acceptor for protein kinases *in vitro*. Both the processing and RNA-binding activities of p54 are enhanced by phosphorylation and decreased by dephosphorylation. In addition, the enzyme is activated by the oxidized form of glutathione and inhibited by the reduced form, whereas other redox reagents that were tested showed no effect. Kinase treatment of p54 prior to oxidation by glutathione resulted in highest levels of activation, suggesting that phosphorylation and redox state act together to control p54 activity *in vitro* and possibly also *in vivo*.

INTRODUCTION

Chloroplasts, the site of photosynthesis, have a central role in the biochemistry and genetics of plant cells. They contain their own DNA and a full complement of proteins involved in all stages of plastid gene expression. Post-transcriptional events are thought to play a major regulatory role, as reflected by the complex array of RNA maturation mechanisms, i.e. internal cleavage of polycistronic transcripts, *cis*- and *trans*-splicing, RNA editing and processing of both the 5′- and 3′-transcript ends (1–7). Processing at 3′-ends has a role in RNA stability in many systems (for review, see e.g. 8–10) including plastid RNAs (11,12). Both structural sequence elements within the 3′-region of plastid transcripts and their cognate binding proteins have been successfully isolated (11,13–18; for review see 12,19).

It is well-established that RNA-binding proteins in nuclear systems can be subject to control by phosphorylation (20–24). This was shown also to be the case for a 28 kDa chloroplast RNA-binding protein from spinach (28RNP), which is required for *petD* 3′-end processing (25–27). Similarly, a plastid protein complex from *Chlamydomonas reinhardtii*, which binds to *psbA* 5′-RNA sequences and has been implicated in translational control, is regulated by phosphorylation (28). Furthermore, the RNA-binding activity of this plastid RNP complex responds to changes in redox potential mediated by dithiothritol (DTT) and thioredoxin (29).

In previous work an RNA-binding protein was purified from mustard (*Sinapis alba*) chloroplasts (30) and identified as a 54 kDa endoribonuclease involved in 3′-end formation of *trnK* and *rps16* precursor transcripts *in vitro* (31). This protein, referred to as p54, was shown to interact with regions containing the conserved 7mer motif UUUAUCU (16).

Here we show that *in vitro* both the RNA-binding and processing activities of p54 can be modulated by phosphorylation and the redox-reactive SH reagent glutathione, but not by DTT and thioredoxin reported to affect other plastid RNPs (25,26,28,29). This suggests that more than a single mechanism may exist for the control of post-transcriptional gene expression by phosphorylation and redox poise in these organelles *in vivo*.

MATERIALS AND METHODS

Plasmid DNA

Plasmid pSPTH80/40 contains the *trnK* 3′ region and has been previously described (30). The *rps16* gene 3′ region is represented by plasmid pSPTS374 as described (31). Plasmids pSPTT317 and pSPTES358 contain the *trnQ* and *trnH* 3′ regions (16) and plasmid pSPTHT150 represents the *psbA* 3′-region (30).

In vitro transcription

In vitro transcripts were synthesized by T3 and T7 RNA polymerases from linearized plasmid DNA and were prepared as described (30).

Purification of the endoribonuclease p54

The p54 was purified from chloroplasts of light-grown mustard seedlings (*Sinapis alba* L.) by chromatography on DEAE–cellulose, heparin–Sepharose and poly(U)–Sepharose as described (31). Protein was determined using the Bradford assay (32) and by comparative gel scanning after silver staining (33).

RNA–protein crosslinking by UV light

^{32}P-labelled RNAs were synthesized *in vitro* by T3 and T7 RNA polymerases from linearized plasmid DNA and were then crosslinked with proteins in 30 mM Tris–HCl pH 7.0, 50 mM KCl, 50 mM $MgCl_2$, 25 µg/ml poly(C) as described (31). RNA-bound proteins were analyzed on 10% SDS–polyacrylamide gels.

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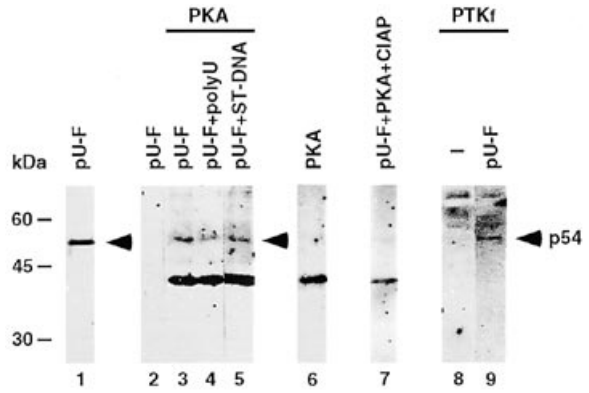

Figure 1. *In vitro* phosphorylation of p54. Purified p54 after poly(U)–Sepharose chromatography (pU-F) was incubated in the presence of [γ-^{32}P]ATP without (lane 2) or with the catalytic subunit of bovine heart kinase (PKA; lanes 3–5 and 7), or with a partially purified plastid kinase (PTK_f; lane 9) for 30 min at 30°C. Lanes 6 and 8, phosphorylation patterns of the two kinase preparations without p54. Lanes 4 and 5, p54 preincubated with either 1 µg poly(U) or 10 µg salmon testis DNA (ST-DNA). Lane 7, PKA phosphorylated p54 with subsequent dephosphorylation with calf intestine alkaline phospatase (CIAP). Proteins were resolved on a 10% SDS–polyacrylamide gel. Lane 1, control lane showing silver-stained p54 after poly(U)–Sepharose chromatography. The location of p54 is indicated by arrowheads.

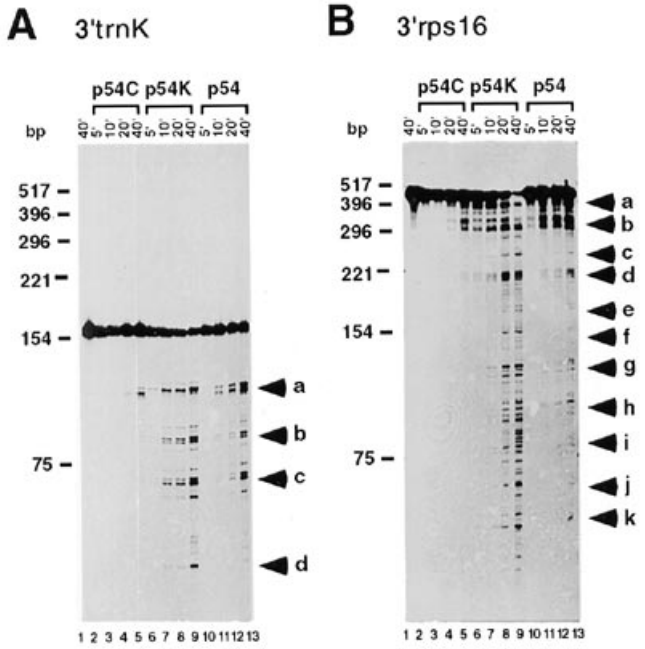

Figure 2. Phosphorylation affects the *in vitro* processing activity of purified p54. ^{32}P-labelled *trnK* (**A**) and *rps16* (**B**) *in vitro* 3′ transcripts were incubated with the CIAP-treated (p54C; lanes 2–5), PKA-treated (p54K, lanes 6–9) or untreated p54 (lanes 10–13) for the times (min) indicated at the top. Lane 1, control lane with no protein. Processing products are marked by lower case letters.

In vitro processing of RNA

RNAs spanning the *trnK* or *rps16* 3′ regions were incubated with 5 µl purified p54 (~8 ng protein) in the presence of 1 µg poly(C) in a final volume of 20 µl using the binding conditions outlined above. After 30 min at room temperature, the RNA was extracted with phenol/chloroform, ethanol-precipitated, and analyzed on 6% sequencing gels.

In vitro phosphorylation and dephosphorylation of p54

Ten microlitre samples of purified p54 (16 ng) were added to the binding buffer containing 5 µCi [γ-^{32}P]ATP and either 2 µg (38 U/mg) of the catalytic subunit of bovine heart kinase (PKA, Sigma) or 0.5 µg of a partially purified chloroplast kinase (PTK_f). The mixture was incubated in a total volume of 50 µl at 30°C for 30 min. Phosphorylated proteins were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. For use in RNA processing or UV crosslinking experiments, p54 was phosphorylated as described above, except in the presence of 0.15 mM unlabelled ATP and without [γ-^{32}P]ATP. The reaction was terminated by overnight dialysis against standard binding buffer without $MgCl_2$.

The p54 protein (10 µl; 16 ng) was dephosphorylated in binding buffer containing 8 mU calf intestine alkaline phosphatase (CIAP, Boehringer Mannheim). The reaction was incubated in a total volume of 50 µl for 30 min at 30°C and subsequently dialysed against binding buffer without $MgCl_2$. Control p54 samples were treated in the same way, but neither ATP, kinase nor CIAP were present.

In vitro redox assays of p54

RNA binding and processing assays were carried out with standard binding mixtures that were pretreated for 10 min at room temperature with 20 mM either of the oxidants menadione (K3, Sigma), cystine (CysCys, Sigma), or oxidized glutathione (GSSG, Sigma), or with the reductants β-mercaptoethanol (EtSH, Sigma), dithiothreitol (DTT, Sigma), cysteine (CysSH, Sigma), or reduced glutathione (GSH, Sigma) before the addition of labelled RNA. *Escherichia coli* thioredoxin (5 µM; MBI Fermentas) was reduced with a 5000 molar excess of DTT or oxidized with a 5000-fold molar excess of menadione, respectively, prior to the individual experiments. In redox-reversibility assays, p54 was preincubated for 10 min with 20 mM of either oxidized or reduced glutathione and subsequently treated for additional 10 min with an equimolar amount of the indicated redox reagent.

RESULTS

Phosphorylation of endoribonuclease p54

We have previously shown that chloroplast p54 plays a role in the 3′-end processing of *trnK* and *rps16* precursor transcripts (31). To test whether p54 can be phosphorylated *in vitro*, we incubated the purified protein with [γ-^{32}P]ATP in the presence or absence of a protein kinase. As shown in Figure 1, lane 2, no autophosphorylation of p54 could be detected, demonstrating that p54 has no kinase activity. Following incubation with a partially purified serine/threonine kinase from mustard chloroplasts (Fig. 1, lane 8) (PTK_f; S. Baginsky, K. Tiller and G. Link, unpublished), a labelled band at 54 kDa was revealed, indicating that p54 was phosphorylated *in vitro* (Fig. 1, lane 9). When p54 was treated with the catalytic subunit of bovine heart kinase (PKA), this again resulted in a radioactive signal at 54 kDa (Fig. 1, lane 3). However, in contrast to the 28RNP from spinach chloroplasts (24), preincubation of p54 with either poly(U) (Fig. 1, lane 4) or DNA from salmon testis (Fig. 1, lane 5) did not further stimulate its phosphorylation.

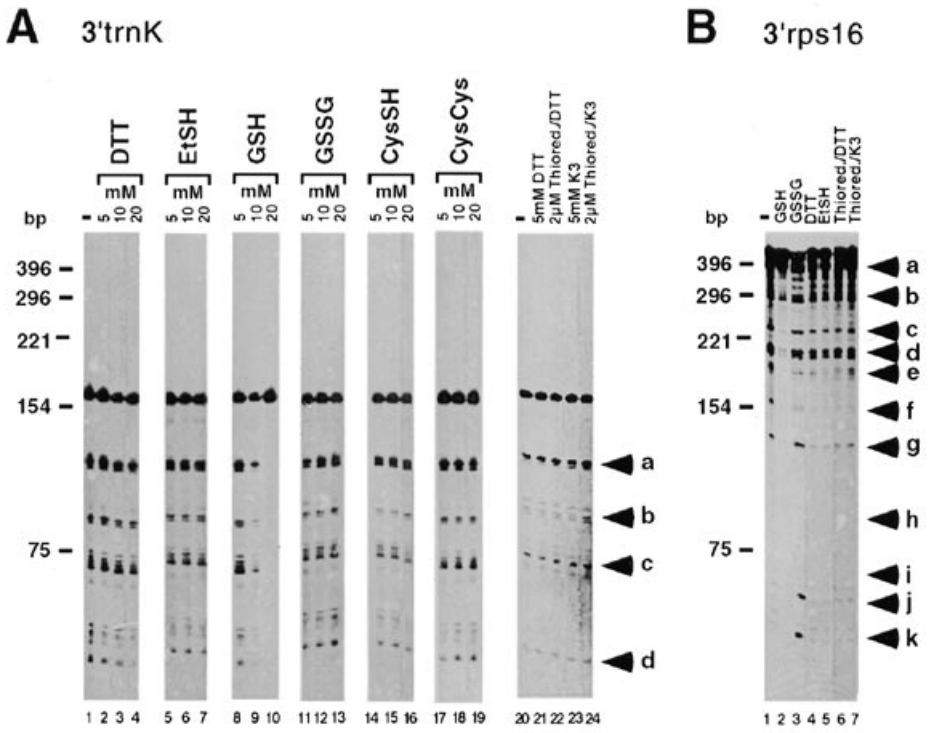

Figure 3. *In vitro* processing activity of p54 in the presence of redox reagents. (**A**) Processing of *trnK* 3′-precursor RNA. Reactions as in Figure 2, containing untreated p54 (lanes 1 and 20) or p54 treated for 10 min with either dithiothreitol (DTT, lanes 2–4 and 21), β-mercaptoethanol (EtSH, lanes 5–7), reduced (GSH, lanes 8–10), or oxidized glutathione (GSSG, lanes 11–13), cysteine (CysSH, lanes 14–16), cystine (CysCys, lanes 17–19), reduced (lane 22) or oxidized thioredoxin (lane 24), or the oxidant menadione (K3, lane 23) at concentrations indicated above each lane. (**B**) Processing of *rps16* 3′-precursor RNA. The protein was untreated (lane 1) or treated with reduced (GSH; lane 2) or oxidized glutathione (GSSG; lane 3), DTT (lane 4),β-mercaptoethanol (EtSH; lane 5), reduced (lane 6) or oxidized thioredoxin (lane 7) before RNA processing assays.

To test whether the phosphorylated form of p54 is a substrate for dephosphorylation by calf intestine alkaline phosphatase (CIAP), labelled p54 was treated after overnight dialysis with CIAP. This experiment resulted in a significant reduction in the amount of labelled p54 and PKA (Fig. 1, lane 7), indicating that indeed phosphorylated p54 is dephosphorylated by CIAP treatment.

Phosphorylation affects p54 processing activity but not specificity

To test if the RNA processing activity is modulated by p54 phosphorylation, we analyzed the products generated by incubation with either the untreated (p54), the phosphorylated (p54K) or the dephosphorylated (p54C) protein.

As previously shown (31), *in vitro* processing of *trnK* 3′-RNA by p54 resulted in four sets of RNA cleavage products in the range of 115, 90, 65 and 45 nt (Fig. 2A, a–d). Cleavage of *rps16* 3′-RNA gave products in the range of 390, 300, 235, 215, 190, 160, 140, 80, 75, 60 and 40 nt (Fig. 2B, a–k). Dephosphorylation of p54 nearly abolished the processing activity with either *trnK* or *rps16* RNA (54C; lanes 2–5), whereas the phosphorylated form (p54K; lanes 6–9) revealed higher activity than untreated p54 (lanes 10–13). However, except for a small (1 nt) shift of the major band at ‘a’, the sizes of the cleavage products remained mostly unaffected. The intermediate activity of the untreated p54 suggests that the protein is already phosphorylated to a certain degree in its native state upon isolation from chloroplasts, and its activity can be further stimulated by kinase treatment *in vitro*. Furthermore, the phosphorylation-dependent activation or inhibition could be partially reversed by treatment of p54K with phosphatase or p54C with PKA prior to the processing assay (Fig. 5, lanes 7 and 15). Hence, together these data indicate that the phosphorylation state modulates the activity, but not cleavage specificity, of 3′-end processing.

The endonuclease activity of p54 is modulated by redox state

To determine if the RNA processing activity was affected by redox state, purified p54 was incubated with either reducing or oxidizing reagents and then analyzed for *trnK* processing activity (Fig. 3A). Neither the reductants DTT or β-mercaptoethanol (EtSH) (Fig. 3A, lanes 1–7), nor cysteine (CysSH; lanes 14–16) and cystine (CysCys; lanes 17–19), showed any significant effect. However, 10 mM of the reduced form of the thiol tripeptide glutathione (GSH) decreased the processing activity (Fig. 3A, lane 9) and 20 mM completely abolished it (Fig. 3A, lane 10). Incubation of p54 with the oxidized form of glutathione (GSSG) led to a slight increase in activity (Fig. 3A, lanes 11–13). Thioredoxin, another possible redox regulator, was tested in its reduced form in the presence of DTT (Fig. 3A, lanes 21 and 22), or in its oxidized form in the presence of menadione (K3) (Fig. 3A, lanes 23 and 24). However, neither was found to modulate the p54 processing activity. This suggests that a redox regulatory site exists on p54, which may be accessible for binding by the tripeptide glutathione only.

When the various redox reagents were tested in p54 processing experiments with *rps16* 3′-RNA (Fig. 3B), results similar to those with *trnK* 3′-RNA were obtained Again, GSH decreased (lane 2)

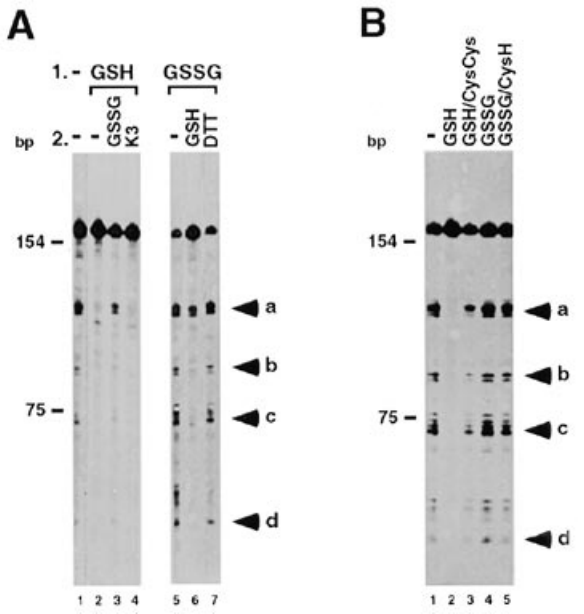

Figure 4. Glutathione specifically affects p54 processing activity. (**A**) Redox reversibility of p54 processing activity with *trnK* 3′-RNA. Lane 1, control lane with untreated p54. Lanes 2–4, p54 incubated with reduced glutathione (GSH) alone (lane 2) or subsequently treated with either oxidized glutathione (GSSG, lane 3) or menadione (K3, lane 4) before processing. Lanes 5–7, p54 treated with oxidized glutathione (GSSG) alone (lane 5) or subsequently treated with either GSH (lane 6) or DTT (lane 7). The band migrating slightly ahead of band a in lanes 2, 4 and 6 was not detected in other experiments. (**B**) Glutathione SH-groups control p54 processing activity. Reactions contained untreated p54 (lane 1), GSH-reduced p54 (lane 2), and GSSG-oxidized p54 (lane 4). Lane 3, GSH preincubated with cystine; lane 5, GSSG treated with cysteine before addition of p54.

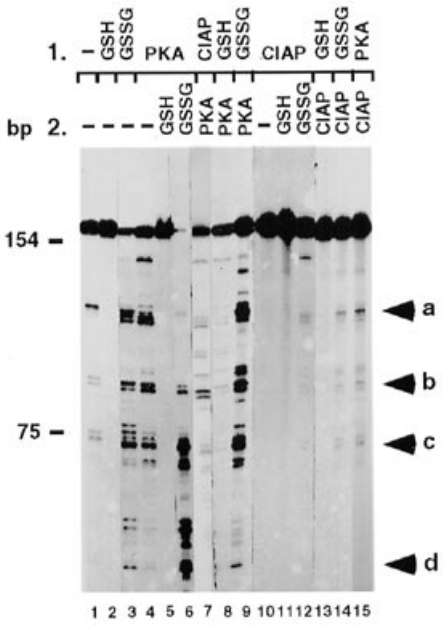

Figure 5. Phosphorylation and redox state together control p54 processing activity. The purified p54 from chloroplasts was pretreated as indicated in the first line at the top (1) and then as listed in the second line (2). Then it was added to processing reactions containing *trnK* 3′-RNA. '–', no treatment of p54; PKA, phosphorylation by protein kinase A; CIAP, dephosphorylation by alkaline phosphatase; GSH, GSSG, treatments with the reduced and oxidized forms of glutathione (20 mM), respectively.

and GSSG slightly increased the processing activity (lane 3), and none of the others showed any significant effect (lanes 4–7).

We next tested the reversibility of the glutathione effects on the p54 processing activity. As shown in Figure 4A for *trnK* 3′-RNA, the decrease in p54 endonucleolytic activity by GSH (lanes 1 and 2) could at least partially be reversed by subsequent treatment with GSSG (lane 3), but not with the oxidant menadione (lane 4). Conversely, the stimulation of activity by GSSG (Fig. 4A, lanes 1 and 5) was reversed by GSH (lane 6) but not by the reductant DTT (lane 7). Although neither cysteine (CysSH) nor cystine (CysCys) had any effect on their own (Fig. 3), they were found to modulate that of glutathione (Fig. 4B). When GSH was pretreated with cystine (CysCys) prior to incubation with p54 and RNA, this resulted in partial recovery from the inhibitory GSH effect (lanes 2 and 3). Pretreatment of GSSG with cysteine (CysSH) led to a small but significant decrease in activity (lanes 4 and 5). Together, these results indicate that the processing activity of p54 is specifically modulated by glutathione.

The temporal order of phosphorylation and redox treatments is important for 3′-RNA processing activity of p54

To test whether phosphorylation and redox state may act together in regulating the p54 activity, we carried out *trnK* 3′-RNA processing experiments with p54 that had been pretreated in various combinations (Fig. 5). Reduced p54 (lane 2; GSH) was inactive and could be partially reactivated by phosphorylation (lane 8; GSH/PKA), but not by dephosphorylation (lane 13; GSH/CIAP). Oxidized enzyme (lane 3; GSSG) was at least as active as the untreated control (lane 1) and was not appreciably affected by phosphorylation (lane 9; GSSG/PKA), but was strongly inhibited by dephosphorylation (lane 14; GSSG/CIAP). Oxidation of p54 followed by phosphorylation (lane 9) also resulted in processing activity comparable to that by phosphorylation alone (lane 4; PKA). This activity (lane 4) was no longer detectable upon subsequent reduction (lane 5; PKA/GSH), suggesting that GSH is always inhibitory to p54, regardless of the order of addition.

Incubation of phosphorylated p54 (lane 4) with oxidized glutathione (lane 6; PKA/GSSG) led to a higher activity than the reverse order of treatments (lane 9; GSSG/PKA). Incubation of p54 with CIAP/GSSG in either order (lanes 12 and 14) resulted in only weak activity, although not quite as low as with CIAP alone (lane 10). This weak residual activity might be due to incomplete dephosphorylation by CIAP in the presence of GSSG.

These experiments suggest that both phosphorylation and oxidation together determine the level of p54 activity, and that the temporal order of treatments is important. Initial phosphorylation followed by GSSG treatment (lane 6) is the most effective combination that gives maximal processing activity.

The RNA binding and processing activities of p54 respond similarly to phosphorylation and redox potential

To find out whether the RNA-binding activity of p54 is affected by phosphorylation, UV crosslinking experiments were carried out with p54 and *trnK* or *rps16* 3′-RNAs (Fig. 6A). Preincubation of p54 with CIAP almost completely abolished the RNA-binding signal (Fig. 6A, lanes 2 and 5). Treatment with PKA gave a more

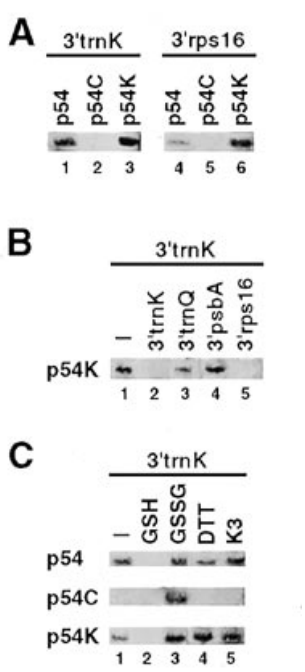

Figure 6. RNA-binding and processing activities of p54 are similarly regulated. (**A**) Phosphorylation controls the RNA-binding activity. UV crosslinking of purified chloroplast p54 with labelled *trnK* (lanes 1–3) and *rps16* (lanes 4–6) 3′-end RNAs. The protein was either untreated (p54, lanes 1 and 4), dephosphorylated (p54C, lanes 2 and 5), or phosphorylated (p54K, lanes 3 and 6). (**B**) Phosphorylation does not affect the specificity of RNA-binding. UV crosslinking assays were carried out with labelled *trnK* 3′-RNA alone (lane 1) or in the presence of excess unlabelled 3′-RNAs as indicated. (**C**) Redox control of p54 RNA-binding activity depends on phosphorylation state. UV crosslinking assays with *trnK* 3′-RNA and untreated (p54), dephosphorylated (p54C) or phosphorylated (p54K) enzyme. Reactions were incubated without ('–') or with the redox reagents indicated at the top.

intense binding signal (lanes 3 and 6) than untreated p54 (lanes 1 and 4). Hence, the binding activity of p54 reponds similarly to changes in phosphorylation state as the processing activity.

To test whether the specificity of RNA-binding by p54 is altered by phosphorylation (p54K), competition experiments were carried out with a 100-fold excess of unlabelled competitor RNAs (Fig. 6B). As for the untreated p54 (16,29), the presence of excess *trnK* and *rps16* 3′-RNAs resulted in disappearance of the binding signal at 54 kDa signal (Fig. 6B, lanes 2 and 5). The *psbA* 3′-RNA partially decreased its intensity (lane 4), and *trnQ* RNA showed little if any competition effect (lane 3). These results indicate that the specificity of RNA binding by p54 is not affected by phosphorylation.

To study the influence of the redox state on the RNA-binding activity, the untreated (p54), phosphorylated (p54K) or dephosphorylated (p54C) protein forms were incubated with redox reagents and then subjected to UV crosslinking assays (Fig. 6C). Neither DTT nor menadione (K3) were found to significantly affect the binding activity (lanes 4 and 5) compared to reactions without redox reagents (lane 1). However, GSH completely abolished the activity (lane 2) and GSSG enhanced the activity of both p54 and p54K and partially restored that of p54C. These results demonstrate that the RNA-binding activity of p54 responds to redox reagents and phosphorylation in much the same way as the processing activity. A summary of the data presented in Figures 1–6 is given in Table 1.

Table 1. Post-translational modifications modulate the activity of the p54 endoribonuclease

Modification	Treatment	Effect
Phosphorylation	(PKA)	+ +
Dephosphorylation	(CIAP)	– – –
Reduction	(GSH)	– – –
Oxidation	(GSSG)	+
Phosphorylation/reduction	(PKA/GSH)	– – –
Phosphorylation/oxidation	(PKA/GSSG)	+ + +
Reduction/phosphorylation	(GSH/PKA)	– –
Oxidation/phosphorylation	(GSSG/PKA)	+ +
Dephosphorylation/reduction	(CIAP/GSH)	– – –
Dephosphorylation/oxidation	(CIAP+GSSG)	– –
Reduction/dephosphorylation	(GSH/CIAP)	– – –
Oxidation/dephosphorylation	(GSSG/CIAP)	– –

The positive (+) or negative (–) response of p54 to the various treatments as detailed in Figures 1–6 is indicated by the number of symbols.

DISCUSSION

In the present study we have obtained results indicating that both phosphorylation and redox state influence the activity of p54, a plastid endoribonuclease involved in RNA 3′-end processing (31). Protein phosphorylation has been reported to be widespread in chloroplasts (34) and seems to affect proteins of many different functions, including those involved in organellar gene expression. Known examples of the latter are the sigma-like transcription factors (SLFs) (35), 3′-RNA binding protein 28RNP (26) and the 5′-binding translational control complex (28) Interestingly, these proteins were all found to be inhibited by phosphorylation and activated by dephosphorylation. The opposite seems to be true for p54, however, where phosphorylation resulted in elevated levels of both processing and RNA-binding activity and dephosphorylation completely inhibited these activities. Furthermore, p54 phosphorylation is not stimulated by DNA or RNA, as was reported for 28RNP (25), neither is it dependent on ADP (not shown), as was found for the translation control complex (28). Together these differences may indicate the existence of multiple pathways for phosphorylation control of plastid gene expression, or, alternatively, of different branches of a single signaling pathway. In addition, other post-translational modifications could be involved in the differential responses of these various proteins to changes in phosphorylation state.

For instance, it was recently shown that the 5′-RNA binding activity of the translational control complex from *Chlamydomonas* is enhanced by the reductant DTT and reduced thioredoxin (29). In our present work we have provided evidence that the activity of p54 can be specifically modulated *in vitro* by glutathione rather than DTT or any of several other reductants. In addition, p54 is inhibited rather than activated by the reduced sulfhydryl form (GSH) and is slightly activated by the oxidized disulphide form (GSSG) of glutathione. Hence, although redox regulation is a common mechanism for the control of plastid gene expression at various levels, the details can vary considerably.

Glutathione is a multifunctional thiol peptide, which appears to be essential in animal and plant cells. Apart from its role as a major sulphur sink, it is involved in a number of important processes, including protection against oxidative stress, detoxification of xenobiotics and regulation of gene expression in response to environmental stress (36–43). In plants, chloroplasts are the

major site of glutathione synthesis (39,40). The glutathione concentrations reported for isolated chloroplasts are 5–10 times lower than those found to be effective in our p54 binding and processing assays *in vitro* (39). A possible explanation for this difference might be the loss of an additional factor in the *in vitro* system. However, we did not observe an increase in glutathione sensitivity with less purified p54 preparations (data not shown). In addition, the *in vitro* glutathione concentration of the site of p54 action may be different from that measured for entire chloroplasts. The intracellular balance of the oxidized and reduced forms is maintained by the mostly plastid-located enzyme glutathione reductase (40). GR is a key enzyme in the protection mechanism against photooxidative stress, the glutathione–ascorbate cycle (44), and both its biosynthesis and activity are regulated by environmental stress conditions (40).

The 5′-RNA binding activity of the translational control complex from *Chlamydomonas* was found to be antagonistically regulated by phosphorylation and reducing reagents (28,29). In our experiments with p54, we also find antagonistic effects, yet in the opposite direction. The most efficient activation of p54 was obtained by phosphorylation prior to oxidation by GSSG. Phosphorylation might be a prerequisite for further activation of p54 by oxidized glutathione, e.g. by mediating a conformational change that promotes specific SH group modification.

It is notable that p54 cleaves at the intergenic region of precursor transcripts that originate from the *trnK* promoter and contain both *trnK* and *psbA* sequences (16,45,46). As a result, two classes of *psbA* transcripts with different 5′-sequences exist, one being generated by this cleavage reaction, the other by transcription from the own promoter of the *psbA* gene (16,47). These different 5′-regions might be starting points for translation control as well as for post-translational modifications (28,29,48–53). Hence, apart from its role in *trnK* 3′-RNA maturation, p54 (and possible associated proteins) may play a role in the stability and translation of the *psbA* RNA.

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